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Role of Antioxidant Enzymes in Acclimatization of *Ceropegia spiralis* Wt., *C. pusilla* Wt. and Arn. and *C. juncea* Roxb.

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Abstract: *The enzyme activities super oxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (As-POX), peroxidase (POX) and glutathione reductase (GR) and phenolic compounds were studied in tuber and shoot tissues from Ceropegia spiralis, C. pusilla and C. juncea during acclimatization under three different environmental conditions: open field, (35˚C-40˚C), green house (25˚C-30˚C) and in culture room (20±2˚C). Significant increase in SOD activity in tubers was discernible with increase in temperature. It is interesting to note that, CAT and GR activities in shoots and tubers differed contrastingly in their patterns with reference to temperature increase wherein a significant reduction in CAT and increase in GR activity were associated with increased tempereture. The variation was more prominent in the shoot tissues rather than in tubers. However, there was not much significant difference among the species with regard to treatments in tubers. Similarly As POX activity, POX activity and phenolics were significantly lower at in vitro conditions both in shoots and tubers and in green house conditions the same were found to be increased.*

Keywords: *High temparature, Super oxide dismutase, Catalase, Peroxidase, Ascorbate peroxidase, Glutathione reductase and Phenols.*

1. INTRODUCTION

Medicinal plants serving as natural sources of antioxidants are widely accepted and being studied. The genus *Ceropegia* L. is the largest genus of the tribe Ceropegiaeae with more than 200 species distributed only in tropical and sub tropical regions of the Old World, ranging from the Spanish Canary Islands in the West, through Central, Southern, and Northern Africa, Madagascar, Arabia, India, South Asia to Northern Australia in the East (Good 1952 and Bruyns 2003).

In India, 55 species are found (Murthy et al. 2012) of which 38 species occur in Western Ghats (Yadav and Mayur 2008). Many species of the genus *Ceropegia* have now been added to the list of Indian endangered plants (BSI 2002). Several species within this genus are rare and endangered, for example *Ceropegia spiralis, C. pusilla* and *C. juncea*. The major threats to these plants are habitat destruction and that edible tubers being eaten by local tribes' people. Due to the elaborate flower forms and ornamentation, several species have horticultural value (CITES 2007). In most of the Indian *Ceropegia* species, the starchy tubers are prone to fungal infections and decay which is a major problem in their maintenance. Overexploitation of *Ceropegia* species for these tubers by humans and various animals, endemism, small and localized populations and severe anthropogenic pressures on the forest land have therefore caused their decline in the wild; the influence of human activities is intense especially in the northern zone of Western Ghats which has been subjected to rapid urbanization in the recent past hence, the species needs conservation. The micropropagated plants of *Ceropegia* spp. are susceptible to higher temperature while hardening. For that matter, all tissue cultured plants the mortality rate is directly proportional to temperature.

Much of the injury to plants exposed to heat is connected with oxidative damage at the cellular level (Foyer and Noctor 2003). The damage of the electron transfer system results in the formation of reactive oxygen species -superoxide anion, singlet oxygen, hydrogen peroxide and hydroxyl radicals). If there is a serious imbalance in any cell compartment between the production of ROS and antioxidant defense, oxidative stress and damage occurs (Mittler 2002). ROS accumulation initiates chain reactions in which superoxide dismutase (SOD) catalyzes the dismutation of O_2^- radicals to

molecular O_2 and H_2O_2 (Meloni *et al.* 2003). The H_2O_2 is then detoxified in the ascorbate-glutathione cycle (Mittler 2002), which involves the oxidation and re-reduction of ascorbate and glutathione through the ascorbate peroxidase (As POX) and glutathione reductase (GR) action (Noctor and Foyer 1998). The protective role of antioxidant enzymes under increasing temperature was studied by Almeselmani *et al.* (2006). The antioxidant action of phenols resides mainly in their chemical structure. There is some evidence of induction of phenolic metabolism in plants as a response to multiple stresses including antimicrobial reactions. Besides the well-studied antioxidant systems consisting of low-molecular antioxidants and specific enzymes, recent works have begun to highlight the potential role of flavonoids, phenylopropanoids and phenolic acids as effective antioxidants. Phenolics, especially flavonoids and phenylopropanoids, are oxidized by peroxidase and act in H_2O_2 scavenging and phenolic/ASC /POX systems.

However, information on the activity of these enzymatic systems is not readily available particularly in the medicinal genus *Ceropegia*. In view of this, some of the antioxidant enzymes (SOD, CAT, As POX, POX, GR and Phenol) in relation to temperature during hardening were studied in three *Ceropegia* species.

2. MATERIALS AND METHODS

2.1. Plant Materials and Sample Preparation

Ceropegia spiralis, C. pusilla and C. juncea (Asclepiadaceae) were collected from Akashaganga of Tirumala hills, Shevaroy hill ranges of Tamilnadu and Directorate of Rice Research campus, Rajendranagar respectively. The acclimatization took place in different flushes with 15d interval. The *in vitro* well rooted and tuberized shoots were washed in running tap water to remove the media traces and transferred to small cups containing acclimatization mixture (vermiculite and coco peat in 1:1). The samples for crude extract preparation were collected from three different environmental conditions, Plants growing in the open field were of 30d old, provided the first set of samples with the mean temperature of 35˚C-40˚C, while the second set of samples were from plants in the green house and 15d old, (tempereture 25˚C-30˚C) and the final set of samples were from test tubes which were maintained for several months in the culture room at 20±2˚C.

2.2. Crude Extract Preparation

Shoot / tuber sample (1.0 g) each were extracted in 10 ml of 0.1M, pH 7.5 M phosphate buffer containing 1mM EDTA, 5% Sorbitol, 0.1% Triton X-100 and then centrifuged at 10,000 rpm for 20 min. The supernatant was used as the enzyme source. All the operations were carried out at 4˚C.

Super oxide dismutase (EC.1.15.1) activity was measured following Dhindsa *et al.* (1981). The sample tubes were illuminated under 15 W fluorescent lamp for 10 min. The tubes lacking enzyme extract but containing the assay mixture were also illuminated and served as control. A non irradiated complete reaction mixture served as blank. Absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as the quantity of enzyme which reduced the absorbency reading to 50% in comparison with the control.

Catalase (EC. 1.11.1.6) was measured according the method of Aebi (1984) in a 3.0 ml reaction mixture containing 1.5 ml of 0.1M phosphate buffer (pH 7.0), 0.5 ml of 75 mM H_2O_2, 0.2 ml crude enzyme extract and 0.8 ml dH_2O. Enzyme activity was calculated on the basis of the amount of H_2O_2 decomposed.

Ascorbate peroxidase (EC.1.10.3.3) was assayed by recording the decrease in optical density to ascorbic acid at 290 nm (Nakano and Asada 1981) in a 3.0 ml reaction mixture containing 1.5 ml of 0.1M phosphate buffer (pH 7.0), 0.5 ml of 3 mM ascorbic acid, 0.1ml of 3.0 mM H_2O_2, crude enzyme extract 0.1ml and dH_2O 0.8ml.

Peroxidase (EC.1.11.1.7) activity was assayed as the increase in optical density due to the oxidation of guaiacol to tetra guaiacol (Castillo *et al.* 1984). The 3.0 ml reaction mixture contained 16 mM guaiacol, 2.0 mM H_2O_2, 50 mM phosphate buffer (pH 6.1) and 0.1 ml crude enzyme extract diluted 10 times.

Glutathione reductase (EC.1.6.4.2) was assayed as per the method of Smith *et al.* (1988). The increase in absorbance was recorded on a spectrophotometer at 412 nm.

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produces blue colored complex.

2.3. Statistical Analysis

All the estimations were performed in triplicates. Values in the figures indicate mean values ± SD, based on two independent experiments and were significantly different as assessed by the analysis of variance (ANOVA) using software Statistics 8.1.

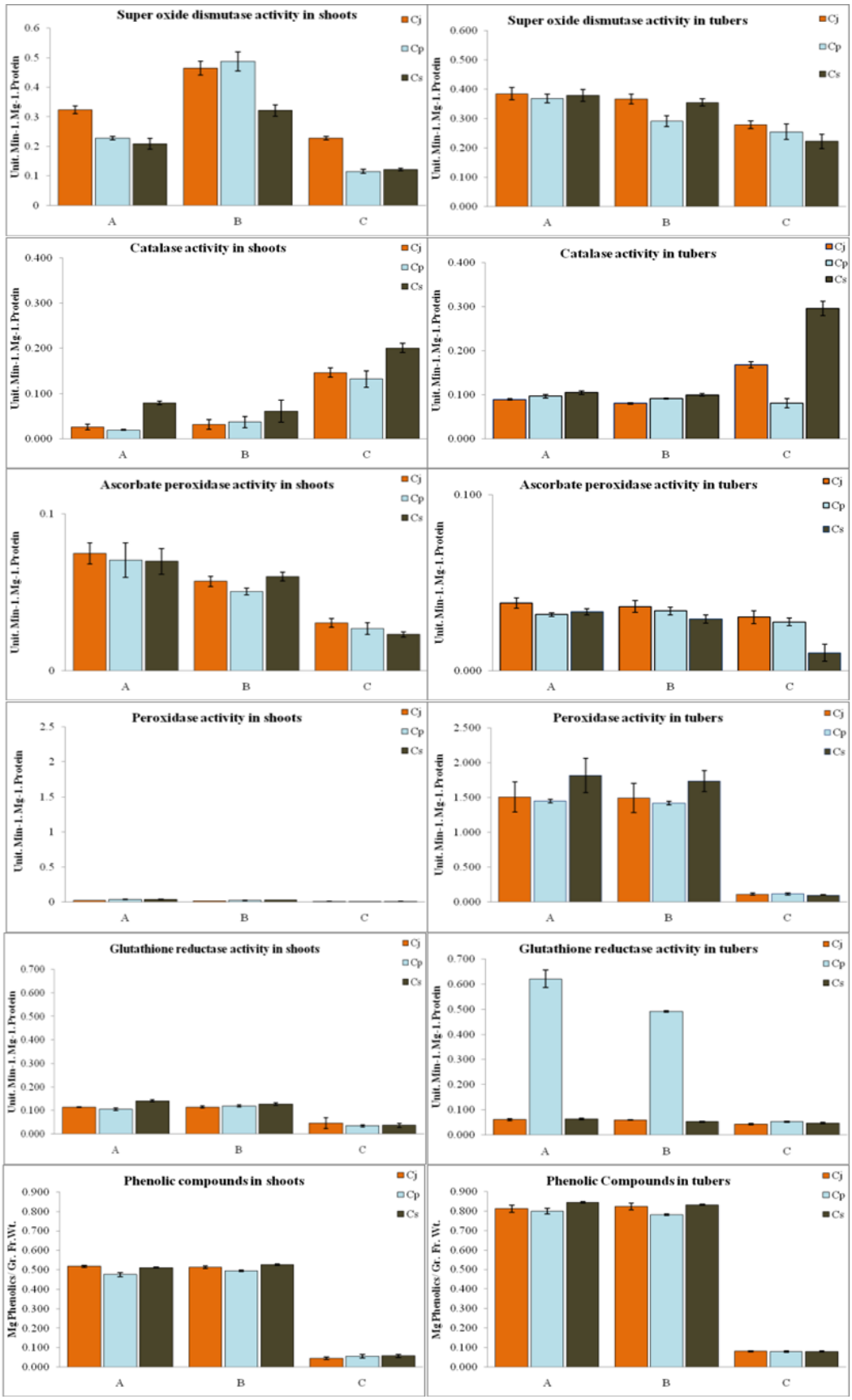
3. RESULTS AND DISCUSSION

The activity of different enzymes varied with samples, species and treatments (Table 1; Fig 1.). The capacity of antioxidants varied with position in relation to irradiance interception (Foyer 1993). The differences in the enzyme activity were more significant in the shoot when compared to roots. The activity of almost all the enzymes was low at 20±2˚C except CAT. The plants growing in test tubes were acclimatized to 20±2˚C. As there was no greater variation in laboratory temperature, plants were steady with the activity irrespective of the enzyme.

Table1. *Variations in anti oxidant enzyme activity with varied temperatures*

Taxa	Sample	Enzyme	Mean activity[§] (Mg/gr. Fr wt.)	Treatments		
				A	B	C
Ceropegia spiralis	Shoots	SOD	0.217	↑	↑*	↓*
		CAT	0.113	↓	↓	↑*
		AsPOX	0.051	↑	↑	↓*
		POX	0.023	↑*	↑	↓*
		GR	0.101	↑	↑	↓*
		Phenolics	0.365	↑*	↑*	↓*
	Tubers	SOD	0.317	↑	↑	↓*
		CAT	0.167	↓	↓	↑*
		AsPOX	0.024	↑	↑	↓*
		POX	1.216	↑*	↑*	↓*
		GR	0.054	↑	↓	↓
		Phenolics	0.585	↑*	↑*	↓*
Ceropegia pusilla	Shoots	SOD	0.277	↑	↑*	↓*
		CAT	0.063	↓*	↓*	↑*
		AsPOX	0.049	↑*	↑	↓*
		POX	0.022	↑*	↑	↓*
		GR	0.086	↑	↑	↓*
		Phenolics	0.342	↑*	↑*	↓*
	Tubers	SOD	0.304	↑	↓	↓*
		CAT	0.089	↑	↑	↓
		AsPOX	0.031	↑	↑	↓
		POX	0.995	↑*	↑*	↓*
		GR	0.388	↑*	↑*	↑
		Phenolics	0.553	↑*	↑*	↓*
Ceropegia juncea	Shoots	SOD	0.339	↓	↑	↓*
		CAT	0.068	↓*	↓*	↑*
		AsPOX	0.054	↑*	↑	↓*
		POX	0.015	↑	↑	↓*
		GR	0.092	↑	↑	↓*
		Phenolics	0.359	↑*	↑*	↓*
	Tubers	SOD	0.342	↑	↑	↓*
		CAT	0.112	↓	↓	↑
		AsPOX	0.035	↑	↑	↓
		POX	1.038	↑*	↑*	↓*
		GR	0.054	↑	↑	↓
		Phenolics	0.572	↑*	↑*	↓*

§- averaged across; **A:** 35˚C -40˚C; **B:** 25˚C -30˚C; **C:** 20±22˚C



Note: Cj: *Ceropegia juncea*; Cp: *C. pusilla*; Cs: *C. spiralis* and A: 35˚C- 40˚C; B: 25˚C-30˚C; C: 20±2˚C

Fig.1. Estimation of antioxidant enzyme activity in different temperature treatments

3.1. Super Oxide Dismutase Activity

In the present study, the SOD activity in the shoots was found to be higher at 25˚C-30˚C while low in tubers at 20±2˚C in all the three species. Xie *et al.* (2011) and Rainwater *et al.* (1996) found that the SOD activity diminished with temperature increase. The study of Dallmier and Martin (1988) reveals that the SOD was thermophobic. With increased temperature increased the SOD activity up to 25˚C-30˚C would be a way removing any excess O_2^- molecules generated. On the other hand, Burke and Oliver (1992) observed that the SOD exhibited a relatively constant activity across a broad range of temperatures. A significant SOD activity was recorded in all species, which indicated that these species had very good scavenging capacity and higher tolerance to heat stress.

3.2. Catalase Activity

As the temperature increased, the catalase activity decreased irrespective of the species both in shoots and tubers. Feierabend *et al.* (1996) observed the photoinactivation of catalase in moderate light under conditions to which plants are adapted. Although the catalases are involved in H_2O_2 scavenging, their turnover being continuous, their steady-state level can be rapidly lowered under stress situations in which translation is inhibited or degradation enhanced (Feierabend *et al.* 1996). The various catalases are however, differentially responsive to light. CAT-1 and CAT-2 mRNA abundance increases upon illumination, whereas CAT-3 shows a rapid and transient decline (Mc Clung 1997). Differential sensitivity of catalases to stress is also observed during temperature extremes. In maize, CAT-3 is more sensitive to temperature than CAT-1 (Havir and McHale 1989). In general, catalase activity is inhibited under low temperature (Omran *et al.* 1980), and heat shock (Delgado *et al.* 1998; Dat *et al.* 1998; Feierabend *et al.* 1992; Hertwig *et al.* 1992). Catalases also exhibited differential response to thermal extremes in maize and *Arabidopsis* (Scandalios *et al.* 1997; Mc Clung 1997).

Catalase does not require reducing power and has a high reaction rate but a low affinity for H_2O_2, thereby removing the bulk of H_2O_2 (Willekens *et al.* 1997). On the contrary, As POX requires reductant (ascorbate) and has a higher affinity for H_2O_2, allowing for the scavenging of small amounts of H_2O_2 in more specific locations (Dat *et al.* 2000). Antioxidant capacity is very much dependent on the severity of the different stresses (Swamy *et al.* 2011). In the present study, we observed the reduction of catalase as the temperature increase but *vice versa* with the As POX.

3.3. Ascorbate Peroxidase Activity

The antagonistic amounts of the catalase and As POX strongly supported the As POX activity mode. Our results are in accordance with Dat *et al.* (2000). The mean activity of As POX in shoots was far superior to the mean activity of the tubers. As the H_2O_2 increases, the As POX activity increases in the cell (Morita *et al.* 1999) and similar increases in the other stresses polyamines were found to be associated with increased As POX. During heat stress, As POX and GR activities are high they have a vital role in protection of plant (Kraus and Fletcher 1994). Lokhande *et al.* (2003) observed that the As POX activity and growth of *A. thaliana* plants increased with higher temperature but not with lower temperature.

3.4. Glutathione Reductase Activity

The GR activity in shoots increased as the temperature increases, but in roots it was very low, whereas great variation in GR activity was found in *C. pusilla* tubers. Similar findings were found in the mustard seedlings (Dat 1998). Gamble and Burke (1984) also observed comparable increase in GR activity in water stressed wheat genotypes. This increase in the GR activity may constitute an adaptive response of *Ceropegia* species to increased temperatures.

3.5. Peroxidase Activity

There was not much variation in the POX activity between 25˚C-30˚C and 35˚C-40˚C but the activity was low at 20±2˚C both in shoots and tubers. This is in full accordance with the studies of Mizobutsi (2010). The activity of POX and catalase, though work independently on ROS, the mode of action of POX on H_2O_2 substrate differs from that of CAT. The POX liberates free radicals rather than oxygen. CAT may scavenge the H_2O_2 produced by POX. Thus, a co-ordinated activity between these two enzymes seems to be critical for temperature related adaptation. Increased temperatures reduced the CAT activity but increased the POX activity concomitantly super oxide radicals and H_2O_2 molecules. CAT plays important role in hardening of *in vitro* raised plants and needs further establishment.

3.6. Phenolics

The phenolics content was naturally higher in the tubers compared to shoots which might alter peroxidation kinetics by modifying the lipid peroxidation order (Arora *et al.* 2000) and showed that they stabilize membranes by decreasing membrane fluidity and hinder the diffusion of free radicals and restrict peroxidative reaction (Blokhina *et al.* 2003). Thermo stability of the plasma membrane is reported to be associated with heat stress and the present results of higher phenolic content in the three species with temperature confirmed the accumulation of phenolics in *Ceropegia* spp.

3.7. Specificity of Enzyme Activity with Respect to Temparature, Species and Tissues

From this study, the activity (increase / decrease) of different enzymes with respect to treatments, species and explant has given obliging information. Irrespective of the treatments, (20±2˚C, 25˚C-30˚C and 35˚C-40˚C) the order of active enzymes were phenolics > POX > SOD = CAT > As POX > GR. The same order was found true with the across each of the three species.

The enzyme dynamics for different temparatures specifically were observed as follows, at 20±2˚C phenols = POX = SOD > CAT = As POX > GR, at 25˚C-30˚C phenolics > POX > SOD = CAT > GR > As POX and at 35˚C-40˚C phenolics > POX > CAT = As POX > GR > SOD.

The species exhibited much similarity in their enzyme kinetics. In *C. spiralis*, the order of importance of enzymes was phenols > POX > SOD > CAT = As POX > GR, whereas in *C. pusilla*, phenols > POX > SOD = CAT = GR > As POX. It was phenols > POX > CAT > SOD = As POX > GR in *C. juncea*.

The variations of enzyme activity were found to be more unsteady in shoots when compared to tubers. As per as the changes in enzyme activity was concerned, the order of enzymes was phenolics > CAT > SOD > As POX > POX > GR found in shoots. Whereas in the tubers, the order was phenolics = POX > SOD > GR > CAT = As POX.

4. CONCLUSION

In conclusion, unsurprisingly, plants possess a well-defined antioxidant defense mechanism to eliminate the perils of free radicals. The results clearly showed that there was differential accumulation of H_2O_2 as well as genotypic variations in H_2O_2-scavenging enzymes in *Ceropegia* species that were grown under naturally different tempereture environments. Such studies might help in recognizing and understanding the differences between an *in vitro* and a greenhouse environment for suitable modifications for conservation under artificial environments. By manipulating the *in vitro* environment, leaves that have greater tolerance to water stress and are photosynthetically competent can be developed as part of the acclimatization process in preparing plantlets for transferring out of culture. Roots formed in culture can be beneficial for enhancing early growth following transfer from culture. The optimum growth rate of deflasked plantlets frequently does not occur until new leaves and roots develop in the greenhouse environment.

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